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INTERACTION OF PROTEINS WITH SMALL MOLECULES

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Introduction

For several decades, an important challenge to biophysical chemists has been to understand the remarkable ability of polypeptide chains to spontaneously fold into stable three-dimensional structures with highly specific biological activities. Although it has been recognized for some time that the three-dimensional conformations of proteins are specified by their amino acid sequences, the factors that determine the stability of a protein structure are still poorly understood, and it is not yet possible to predict the structure of a protein from its sequence alone [1].

The interaction of small molecules with macromolecules of biological systems and with specific receptor sites on surfaces of supramolecular organizations is one of the most extensively studied phenomena in biochemical research. The subject includes a vast range of important biochemical phenomena: the approximation of protein stability and non-covalent forces pertaining to a protein structure; an understanding of the mechanisms of action of enzymes and of regulatory systems which often requires knowledge of the number of binding sites or the strength of binding; the reversible interactions related to the combination of enzymes with substrates and inhibitors and the kinetic phenomena of enzyme-substrate interaction commonly involve reversible as well as irreversible processes; the binding and release of protons by basic and acidic groups in proteins; the association of small cations, especially calcium and magnesium ions, with proteins and nucleic acids; the non-covalent association of protein with fatty acid anions and other compounds containing non-polar groups and protein-lipid interaction; the reversible binding of oxygen, carbon monoxide and other compounds by myoglobins and hemoglobins; the antigen-antibody interaction; protein-protein and protein-DNA interaction and there are probably as many intercellular reactions as well as inter and intra molecular interactions.

The fact that ligand binding processes can frequently be coupled to associated structure changes in the protein has interesting energetic and functional consequences. Ligand induced conformation changes are often an essential part of the formation of the catalytically active complex of enzymes or of the transduction of information or energy. The energy changes involved in these important phenomena are usually due to non-covalent forces and are small compared with those accompanying the formation or breakage of covalent bonds. However, binding equilibria can be measured accurately enough to discriminate between processes which differ only in small energy increments. In fact, the discrimination in terms of energy changes is much finer than any explanation in terms of structure and mechanism. Such studies are, therefore, an essential part of the physicochemical characterization of many biological phenomena ranging from enzyme catalysis and its control, hormone action, membrane transport, and nerve conduction to muscle contraction and other forms of motility.

Since proteins are usually denatured by their interaction with ligands, the denatured states of proteins have attracted increasing attention. This interest arises from the realization that the denatured state is the only experimentally achievable state of a protein that can be taken as an initial reference in considering the mechanism of folding and stabilizing the native protein structure. However, a denatured state can play this role only if it can be associated with a completely unfolded, random coil conformation of polypeptide chain. The latter, as is clear, is an idealized model that is never realized in practice, since real polypeptides have too many groups interacting in very different

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ways with each other and with the solvent. However, it is unclear how far the real situation is from the ideal, and how large an error arises from equating a random coil with the denatured state, particularly when it is being used to estimate the thermodynamic parameters for folding of the native protein structure [2]. A detailed historical review of denaturation studies as part of protein chemistry would need a lot of space. Hence, I shall limit myself only to an enumeration of the existing reviews.

An early comprehensive review was written by Neurath, Greenstein, *et al.* [3]. It was followed by a review by Putnam [4] and two reviews by Kauzmann [5, 6]. Joly has also published a book on protein denaturation [7]. Then, two reviews have been written by Tanford [8, 9] and Brandts [10]. There are also some reviews that deal only with a particular aspect of this phenomenon [11 , 12]. Lapanje has also published an interesting book on the physicochemical aspects of protein denaturation [13]. The thermodynamics of protein denaturation has been the subject of reviews by Privalov and Pfeil [14, 15]. Hinz has also written a very readable review on the microcalorimetry of protein - ligand interaction and protein unfolding [16]. A nice review on the stability of protein structure and hydrophobic interaction was produced by Privalov and Gill [17]. Finally, there are two comprehensive reviews on cold denaturation and on measuring and increasing protein stability by Privalov [18] and Pace [19], respectively.

Denaturation can be brought about in many ways. The main modes and types of denaturation are as follows: thermal denaturation [20, 21]; cold denaturation [18]; pressure denaturation [22, 23]; denaturation by changing pH [24, 25, 26 and 8]; denaturation by organic solvents and solutes [27, 28, 29 and 30]; denaturation by inorganic salts [31]; denaturation by urea and guanidine hydrochloride [32, 33] and denaturation by surfactants [34, 35].

In this review, attention will mostly be paid to urea and guanidine hydrochloride [GuHCl) as well as to a special discussion on surfactant interaction with proteins.

Discussion

Urea is one of the most widely used denaturants. Usually it is efficient only at relatively high concentrations (> 8M). However, even at such high concentrations many proteins are not completely unfolded. The actual extent of unfolding depends also on temperature, pH and ionic strength. Although GuHCl is one of the strongest denaturants, in 6M GuHCl all proteins with an ordered structure usually lose it, and most of them become randomly coiled and do not contain any residual structure.

Urea and GuHCl ($H_2N-\underset{\underset{O}{\|}}{C}-NH_2$ and $H_2N-\overset{\overset{NH_2}{|}}{C}=NH_2^+Cl^-$)

have complicate interactions with proteins. Initial rationalizations focused upon their obvious potential for hydrogen bonding, and they were thought to break protein hydrogen bonds. However, further reflection, plus some experimental data, indicated that they were no more potent in this respect than water. Model compound studies demonstrated that both increase the solubilities of non-polar molecules, including those of the amino acid side chains, in proportion to their accessible surface area [36], diminishing the magnitude of the hydrophobic effect by up to one third. This is probably an indirect result of their effects upon the structural properties of water, resulting from their comparable hydrogen-bonding capabilities, but with different geometries. In confirmation, the denaturant potencies of GuHCl are affected by the nature of the anion according to the Hofmeister series [37].

Diminishing the hydrophobic interaction by one third should be ample to produce the unfolding of proteins, but the observed effects are usually considerably less than those predicted; therefore, there must be additional factors, probably direct interactions with the proteins. Many theories of unfolding have concentrated on specific interactions between denaturants and the protein, explaining unfolding by the possibility of more interactions with the unfolded state. However, it is not clear how specific such interactions could be if concentrated solutions like 6M GuHCl are required, where the denaturant occupies half the volume of the solvent. Nevertheless, interactions with protein are apparent by a variety of techniques, and crystallographic studies of α-chymotrypsin in the presence of GuHCl and urea show a variety of interactions with the folded protein [38]. Urea molecules, but not the charged guanidinium ion, even permeate the interior, occupying small cavities and perturbing somewhat the close-packed interior. Consequently, it is most likely that denaturants such as urea and the guanidinium ion act indirectly by diminishing the hydrophobic interaction in a uniform, predictable manner, and directly interact with both the folded and unfolded states to produce a wide range of effects, depending upon the local geometry of the interacting groups in the protein [39].

A large part of the action of urea, GuHCl and similar compounds is thought to involve a hydrophobic mechanism that favors exposure to the solvent of

non-polar groups in the interior of the protein molecule. In general, aqueous solutions containing a high concentration of these compounds act as better solvents for non-polar substances than does water alone. Studies on the transfer of hydrocarbons used as models for the amino acid side chains from water to 7M urea or 4.9 M GuHCl revealed a favorable Gibbs free energy (ΔG) for the process. Although the transfer requires energy ($\Delta H > 0$) at room temperature it is accompanied by a positive entropy change that overrides the unfavorable enthalpy change [40].

The studies of protein denaturation by various means in aqueous solutions have attracted much attention and are mandatory in a proper evaluation of the thermodynamic quantities of denaturation. One of them is the stabilization Gibbs free energy ΔG°_{D}, defined as Gibbs free energy necessary for converting the protein from its native state to random coil. It is evident that a knowledge of ΔG°_{D} is crucial for understanding the forces instrumental in protein folding as well as the stability of the native state. The other two quantities, the stabilization enthalpy, ΔH°_{D}, and the stabilization entropy, ΔS°_{D}, are also of importance, since they are needed for the complete thermodynamic description of denaturation, and lead to additional insight into the nature of the phenomenon [41, 42].

For many years it has been accepted that the best approximation of random coil polypeptide is a denatured protein in concentrated solutions of GuHCl or urea, while a heat or acid-denatured protein contains too much residual structure to be regarded as a completely unfolded, unstructured state [8]. This conventional view was shaken first when it was shown, by direct calorimetric measurements, that the enthalpies of lysozyme denaturation by heat and GuHCl are identical if the denaturant solvation is correctly taken into account [43]. It was also found that the partial heat capacities of heat and GuHCl denatured lysozyme are indistinguishable.

The importance of the partial heat capacity in specifying the denatured state of proteins follows from the fact that this parameter is a sensitive index of the completeness of protein unfolding. The exposure of the internal non-polar groups to water should result in a heat capacity increment, since the transfer of non-polar compounds to water is associated with a significant increase of the heat capacity. On the other hand, the heat capacity is the temperature derivative of a basic thermodynamic function, the enthalpy. Therefore, the denaturational heat capacity increment determines the temperature dependence of the enthalpy and hence, of the entropy of denaturation, i. e. the parameters that determine the native state stability.

Bigelow and co-worker [44]; Sarfare and Bigelow [45]; Pace and co-workers [48, 49, 50]; Ahmad [51] and Aune and Tanford [52] measured the isothermal unfolding of RNase-A, lysozyme α-lactalbumin, β-lactoglobulin, α-chymotrypsin and trypsinogen under a variety of denaturing conditions using various physical techniques. The overall conclusion of their studies is that different denaturants could produce different denatured states, i.e. conformations with different amounts of secondary and tertiary structures. Only GuHCl and urea gave the most extensively unfolded state, one in which the protein molecule is devoid of all elements of its native conformation and behaves as a random coil. The denatured states obtained in other denaturants are "intermediate" states between native and urea or GuHCl-denatured states.

Supporting evidence that heat-denatured proteins contain residual structure came from the observation, by Aune *et al.* [53], that another cooperative transition occurs when GuHCl is added to thermally denatured RNase lysozyme or chymotrypsinogen after the completion of the thermal transition. Ananthanarayanna *et al.* made a similar observation with bovine β-lactoglobulin [54].

It is also known that inorganic salt denaturants give intermediate denatured states, but the evidence for this conclusion is based only on the observations of intermediate values of the physical properties chosen for study, that is to say, values between those observed for the native molecule and the completely disordered one seen usually in GuHCl or urea [44, 45, 46, 55].

The conformational stability of most naturally occurring globular proteins is surprisingly low, generally 5-15 kcal mol^{-1} [56]. For most industrial applications, the goal is to construct the most stable enzyme possible that still has the desired enzyme activity. Researchers have been trying to increase the stability of subtilisin [57, 58] and have designed and constructed a protein based on a four-helix bundle structure with a conformational stability of 5-22 kcal mol^{-1} [59].

The conformational stability of a globular protein may be defined as the free energy change for the reaction:

$$\underset{\text{(native)}}{\text{folded}} \leftrightarrow \underset{\text{(denatured)}}{\text{unfolded}} \qquad (1)$$

under ambient conditions such as in water at 25°C. Estimates from urea or GuHCl denaturation curves are designated ΔG (H_2O), and estimates from thermal denaturation curves are designated ΔG (25°C). Pace [19] has introduced methods used to measure ΔG (H_2O) and ΔG (25°C), and has described the simplest method used to estimate the difference in stability between

two proteins differing slightly in structure. Such a structural change might be a single change in amino acid sequence made by site-directed mutagenesis, or a change in the structure of a side-chain made by chemical modification. Finally, the most promising methods to increase the conformational stability of proteins are discussed by Pace [19].

Pace has estimated the stability from urea and GuHCl denaturation curves for RNase A and many other globular proteins, the mechanism of unfolding has been shown to approach closely a two-state mechanism where the concentration of partially folded molecules present at equilibrium is small enough to be neglected. (For practical information on measuring and analysing a denaturation curve, see Ref. 60). By assuming a two-state folding mechanism, the fraction of denatured protein F_d, may be calculated using:

$$F_d=(y_n-y_{obs})/(y_n-y_d) \qquad (2)$$

where y_{obs} is the observed variable parameter (e. g. fluorescence intensity, Fig. 1a) and y_n and y_d are the

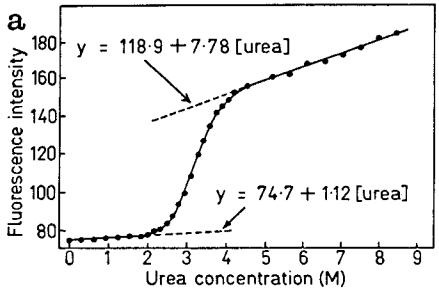

Figure 1a. Urea denturation curve for RNase A in 30 mM formate buffer, pH 3.55 25°C. Fluoresence intensity was measured at 305 mm after excitation at 278 nm. Broken lines and equations are based on a least-squares analysis of the pre-and post-transition baselines.

values of y characteristic of the native and denatured states (obtained as shown in Fig. 1a). The difference in free energy between the folded and unfolded conformations, ΔG, can then be calculated using:

$$\Delta G = -RT \ln [F_d/(1-F_d)] = -RT \ln [(y_n - y_{obs})/(y_{obs} - y_d)] \qquad (3)$$

where R is the gas constant and T is the absolute temperature. In general, ΔG varies linearly with denaturant concentration (Fig. 1b). The method of least-

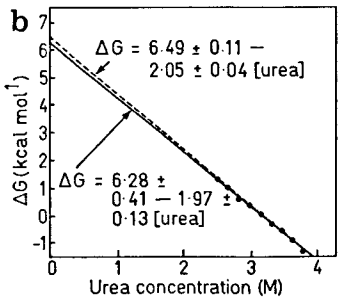

Figure 1b. ΔG (calculated from the measurements in the transition region using Eqn. 3) vs urea concentration. Broken line and corresponding equation (with the standard errors given) were obtained by a least-squares fit of the data to Eqn. 4 taken from Ref. 19.

squares analysis is used to fit the data from the transition region to the equation:

$$\Delta G = \Delta G(H_2O) - m[D] \qquad (4)$$

where ΔG (H_2O) is the value of ΔG in the absence of denaturant, and m is a measure of the dependence of ΔG and denaturant concentration [61]. Currently, linear extrapolation seems the most suitable method available [62].

Lapanje reported on many works on the interaction between proteins and urea and some alkylureas as well as GuHCl [63, 64, 65, 66 and 67]. Pace has also applied many methods for studying protein conformation induced by protein ligand interaction [68, 69, 70, 71 and 72]. Ahmad has also reported on penetrating studies on the denaturation of proteins from urea and GuHCl interactions [73, 74, 75]. Creighton has worked on the detection of folding intermediates using urea-gradient electrophoresis [76] and many studies on the kinetics of folding intermediates [77, 78].

The interactions of proteins with ionic surfactants differ from their interactions with other ligands in two important respects:

1) Ionic surfactants having hydrocarbon-chain lengths of eight or more carbon atoms are the most potent protein denaturants known [79].

2) Unlike all other classes of ligand, except hydrogen ion, they combine with most native proteins in multiple equilibria, i. e. many equivalents per mole of protein. They combine in still larger quantities with proteins that have been unfolded. In fact, they unfold proteins precisely because the difference in binding

equivalence exists.

Thus, the two unique interactions of ionic surfactants with proteins are closely interrelated. The observation by Anson in 1939 that a commercial sample of sodium dodecyl sulphate (SDS) was a potent protein denaturant directed the attention of protein chemists to surfactant interaction. Urea (8M) has been as a protein denaturant, and GuHCl at about 6M has been the denaturant of choice since Tanford showed that, in solutions of the latter, reduced proteins lost all traces of secondary, tertiary, and quaternary structure, and behaved like randomly coiled long-chain synthetic polymers [8]. Dodecyl sulphate, however, produces a maximum unfolding effect, especially of reduced proteins, at concentrations of less than 10^{-2}M, which may actually contain only 10^{-3}M or less of the detergent in the uncombined form. In 1% solutions barely more than 15 moles of detergent per mole of protein are required to produce unfolding of some protein molecules. The ratio for GuHCl under identical conditions is about 40,000.

The binding of surfactants to proteins follows the usual thermodynamic laws of equilibrium. Binding has been found to occur to discrete binding sites and is a function of the free surfactant concentration in equilibrium with the protein. It is influenced by temperature, pH, ionic strength and other environmental factors, which should be controlled. A general discussion of the methods employed to determine binding is provided by Steinhardt and Reynolds [80, 81]. Analysis of the binding data is usually performed as presented by Scatchard [82] and Klotz [83]. In this way, binding sites with different levels of affinity can be revealed and analyzed.

The specific intramolecular structure and amphoteric characteristic of proteins distinguishes them from the synthetic surfactants which are dissociable colloidal electrolytes. Proteins and detergents both contain a balanced proportion of hydrophilic and hydrophobic groups and are thereby made surface active. In the case of proteins, these moieties consist of the polar and nonpolar residues of the amino acid chains distributed along the relatively inert pertide "backbone", while for the ionic surfactant, the hydrophobic moiety is a long or partially cyclic hydrocarbon chain joined to a hydrophilic "head".

In general, surfactants interact rapidly with globular proteins in aqueous media, the initial interaction involves the monomeric surfactant and occurs below the critical micelle concentration (cmc). The interaction usually results in the unfolding of protein (i. e. the protein is denatured) and if the protein is an enzyme the enzymic activity is lost [84, 85 and 86], whereas, in marked contrast, the activation of some proteins can occur, for example Aspergillus niger catalase is activated by SDS [95, 100].

Long-chain fatty acids such as lauric acid or the corresponding surfactant such as sodium n-dodecyl sulphate (SDS) reacts readily with proteins, frequently causing dissociation into subunits and denaturation of the individual poly-peptide chains. The disruption of proteins with SDS has had extensive application and is particularly useful as a prelude to the estimation of molecular weights and subunit stoichiometry by gel electrophoresis.

Physical studies of SDS-protein complexes at high binding levels (about 1.4 g/g) suggest a model of a thin rod of constant diameter and length proportional to the length of the chain [87]. The dimensions are consistent with a helix like conformation with SDS molecules intercalated along the length of the helix. At very low levels of SDS binding, marked conformation changes do not generally occur. It seems that the early stages of binding involve polar interaction of the changed group of SDS and protein at the surface, subsequently, apolar interactions probably play an important role in the SDS-protein complexes [34, 88 and 89]. The interaction of SDS with some proteins at high ionic strengths results in binding of over 1.48 g of SDS/g of protein [34, 94].

It has been demonstrated for a number of surfactants that it is the monomeric species that is bound and not the micellar form, when both ligand and protein are present at relatively low concentrations [90]. It is therefore the free monomeric concentration of the surfactant that determines the amount bound to the protein. Little further binding is observed upon increasing the free detergent concentration beyond the cmc. This also means that the binding of surfactants to proteins has to compete with the self association of surfactant molecules to micelles. This competitive feature of the binding process effectively limits the acquisition of analyzable data to situations where the free energy gain associated with binding to the protein exceeds the free energy gain in micelle formation [91, 93]. The binding of protein molecules to pre-existing surfactant micelles may, however, be possible in some instances.

Preliminary data cited by Tanford, *et al.* [92] indicate that with membrane proteins and lipoproteins, cooperative interactions can be observed above the cmc, and such processes may be accompanied by changes in the state of aggregation of the protein as well. Aspergillus niger catalase also binds to SDS beyond the cmc [94] which necessitates correction for micellar dissociation (demicellization) in order to obtain the true enthalpies of interaction between Aspergillus

niger catalase and "monomeric" surfactant.

Much of the work in the area of the protein-surfactant interactions has been documented by Jones [96, 97]; Takeda [98, 99] and the author [100, 101]. The interaction of sodium n-dodecyl sulphate [SDS) with a range of globular proteins; histones [102, 103]; catalase [104, 105] and glucose oxidase [106] have been investigated by a range of physical methods, including equilibrium dialysis, microcalorimetry, ultracentrifugation, viscometry and spectroscopy. Thermodynamic and kinetic analysis of specific and cooperative binding in terms of Scatchard and Hill equations of interaction of histones and SDS has been investigated [107, 108, 109].

The effect of SDS on the thermal stabilization of the histones H_2A, H_3 (over the range of 30°C to 90°C by temperature scanning spectrophotometry) was estimated from thermodynamic parameters as well as melting temperature. It indicated a compact structure for the histones-SDS complexes. By contrast, these values are inconsistent for dodecyl trimethyl ammonium bromide (DTAB) and more in line with DTAB including the unfolding of the histones, H_2A, H_3, H_2B [110]. The interaction of DTAB with histones has also been investigated by equilibrium dialysis [111, 112] to obtain thermodynamic parameters. The corresponding unfolding enthalpy was estimated which is a good estimation of the cooperativity of the system.

The thermal denaturation of adenosine deaminase has been investigated in the presence of DTAB over the temperature range of 305-355°C by temperature scanning spectrophotometry. The presence of DTAB caused the destabilization of adenosine deaminase resulting in a decrease in the temperature of unfolding with increase in DTAB concentration [113].

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